The cerebellar transcriptome during postnatal development of the Ts1Cje mouse, a segmental trisomy model for Down syndrome.

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Abstract

The central nervous system of persons with Down syndrome presents cytoarchitectural abnormalities that likely result from gene dosage effects that affect the expression of key developmental genes. To test this hypothesis, we have investigated the transcriptome of the cerebellum of the Ts1Cje mouse model of Down syndrome during postnatal development using microarrays and quantitative PCR. Genes present in three copies were consistently over expressed, with a mean ratio relative to euploid of 1.52 as determined by quantitative PCR. Out of 63 three-copy genes tested only 5, 9 and 7 had ratios >2 or <1.2 at postnatal days 0 (P0), P15 and P30, respectively. This gene dosage effect was associated with a dysregulation of the expression of some two-copy genes. Out of a total of 8258 genes examined, the Ts1Cje/euploid ratios differed significantly from 1.0 for 406 (80 and 154 with ratios >1.5 and <0.7, respectively), 333 (11>1.5 and 55<0.7) and 246 (59>1.5 and 69<0.7) at P0, P15 and P30 respectively. Among the two-copy genes differentially expressed in the trisomic cerebellum, 6 homeobox genes, 2 belonging to the *Notch* pathway, were severely repressed. Overall, at P0, transcripts involved in cell differentiation and development were over represented among the dysregulated genes, suggesting that cell differentiation and migration might be altered more than cell proliferation. Finally, global gene profiling revealed that transcription in Ts1Cje mice is more affected by developmental changes than by the trisomic state and that there is no apparent detectable delay in the postnatal development of the cerebellum of Ts1Cje mice.

Introduction:

Down syndrome results from trisomy of human chromosome 21 and is the most frequent genetic cause of mental retardation, occurring in ~1 in 800 newborns. Most cases are full trisomies, and about 300 genes are triplicated (1). This dosage imbalance causes dysfunction of developmental and physiological processes, but there is a puzzling variability among affected individuals in terms of the number of clinical features observed and their severity (2). Therefore, one of the most relevant questions in Down syndrome research concerns the identification of the genes (within or outside of chromosome 21) that contribute both to the phenotype and to phenotypic variability. In addition, we believe that the identification of these genes is essential for the development of new therapeutic approaches. The search for gene targets that may be amenable to therapeutic approaches has accelerated in the past few years with the publication of the human genome sequence and the use of large-scale gene expression studies such as SAGE and microarrays (3,4,5,6,7).

Because of the prominence of mental retardation, the brain has been the subject of particular interest in research on Down syndrome, but a systematic study of gene expression in brain during development, particularly at postnatal stages, is only possible in animal models. Mouse models for Down syndrome are either trisomic for single genes, for several genes or for a large segment corresponding to the distal part of chromosome 16, orthologous to a large portion of human chromosome 21 (8). Two models of segmental trisomy 16 have been generated, the Ts65Dn and the Ts1Cje mice (9,10,11). These models have revealed previously unknown phenotypes that may be relevant to Down syndrome, such as a substantial loss of glutamatergic granule cells in the internal layer of the cerebellum in adult (12,13).

We studied the transcriptome during postnatal development of the cerebellum for two reasons. First, there are massive developmental changes during the first 20 days after birth. During this period, granule cells, which represent about 40% of the total number of neurons of the mature cerebellum, proliferate, migrate and differentiate from the external to the internal layer, and Purkinje cells develop their dense dendritic trees making connections with other cells. Second, gene profiling during postnatal development of the cerebellum in mice is well documented (14,15), Furthermore, previously investigated mutants of the cerebellum could serve as a reference to our study (14).

The Ts1Cje Down syndrome model was used in the present study after backcrossing the mice on to a pure genetic background to reduce variability in gene expression. Ts1Cje mice carry a segmental duplication of the syntenic region orthologous to human chromosome 21 from *Sod1* to *Znf295* including about 95 genes (10,11,8). Differential gene expression was studied

at postnatal day 0 (P0), P15 and P30 in the cerebellum using Affymetrix U74Av2 microarrays. Expression of genes was also measured in parallel by quantitative PCR (qPCR). Two groups of genes have been characterized: one that contains the triplicated genes and shows a consistent gene-dosage effect (~1.5 over expression in the Ts1Cje mice as compared to the euploid) during postnatal development, and a second that contains euploid genes that are significantly differentially expressed (over or under) and are mainly involved in cell differentiation and development.

Results:

Experimental data and analysis: validation.

Using Affymetrix murine genome U74Av2 microarrays, we measured the expression levels of 12 488 genes (half known genes and half ESTs) in cerebellum of Ts1Cje and euploid mice each at P0, P15 and P30 during postnatal development. The mean expression value for each gene was calculated from duplicate experiments performed on the 6 groups of 3 animals each. According to the Affymetrix algorithm, 8287 genes were found to be expressed at least at one time point (see Materials and Methods for details on the filtering step and supplemental data table S1 for a complete list of the 8287 genes). Two statistical analysis were applied to the data: VARAN (VARiability ANalysis of microarray data), a freely available web server performing a signal intensity based analysis of the log2 expression ratios variability deduced from DNA microarray raw data (16) and ANOVA. The parametrization chosen for the ANOVA allowed the selection of genes either involved in normal postnatal development of the cerebellum or differentially expressed in the Ts1Cje as compared to euploid mice at one particular stage of development (α_i and β_i parameters respectively in Table 1 in Materials and Methods).

To validate our experimental data and analysis, we compared genes differentially expressed between P0 and P15 or P15 and P30 (α_i parameters) in either euploid or Ts1Cje mice to genes differentially expressed along normal postnatal development of the cerebellum as reported by Diaz *et al.* (14). Their experimental data, which were obtained on the mouse RIKEN 19-K microarray, were analyzed using ANOVA with the parametrization described in Table 1 in Materials and Methods (αi parameters). Many genes differentially expressed during postnatal development of the cerebellum, such as GABA−α 6 and GABA−δ, myelin oligodendrocyte glycoprotein, and parvalbumin (Supplemental data table S2), belonged to the cell differentiation class: B class in Diaz *et al.*, (14). Discordance between the two studies are

generally the result of differences in the gene content of the two microarrays. We thus concluded that our data were as informative as those of Diaz *et al.*(14).

Gene expression in the Ts1Cje cerebellum: triplicated versus euploid genes from mouse chromosome 16.

Of the 8287 expressed genes, 145 were from mouse chromosome 16, and among them 29 were triplicated in Ts1Cje mice. At P0, P15 and P30, mean expression ratios of triplicated genes between Ts1Cje and controls were 1.66, 1.32 and 1.32 at P0, P15 and P30, respectively, whereas with euploid genes the ratios were 1.08, 1.12 and 1.02 at P0, P15 and P30, respectively (Fig.1). After ANOVA, 12, 6 and 9 triplicated genes were found to be statistically over expressed (α=1%) in Ts1Cje mice as compared to euploid at P0, P15 and P30 respectively (Table 2). Among these genes, four (*Dscr3, HMGP14, Donson, C21orf4*) were over expressed at all three time points.

Quantitative PCR (qPCR) on 78 genes from chromosome 16 (15 euploid and 63 triplicated) showed over expression of the triplicated genes with mean ratios of 1.56, 1.43 and 1.55 at P0, P15 and P30, respectively, whereas the euploid genes from chromosome 16 had ratios very close to 1.0: 1.04, 1.00 and 1.01 at P0, P15 and P30, respectively. (Fig.2 and supplemental data table S3). The 18 control genes mapping to chromosomes other than 16 and described previously (18) showed ratios of 1.02, 1.08 and 1.03 at P0, P15 and P30, respectively. Gene expression of the 33 euploid genes from chromosome 16 and other chromosomes did not reveal any significant correlations between stages. Interestingly, for the triplicated genes there was a highly significant correlation ($p<0.01$) between the P15 and P30 time points. Only a few genes had ratios above 2 (3, 2 and 5 at P0, P15 and P30 respectively) or below 1.2 (2, 7 and 5 at P0, P15 and P30 respectively).

qPCR and microarray measurements were highly correlated ($p<0.01$) for 45 transcripts tested that were present in both microarray and qPCR studies. A single and expected exception was the *Sod1* transcript. The wild type *Sod1* gene is composed of 5 exons, but in Ts1Cje mice a neomycin cassette is inserted into exon 3, thereby generating a shorter transcript with only exons 1, 2 and 5 (17). DNA probes on microarrays detected this new transcript, but qPCR did not, since the primers span exons 3 and 4 (18).

Gene expression in the Ts1Cje cerebellum: euploid genes from all chromosomes: statistical analysis and validation by qPCR:

In addition to the triplicated genes from chromosome 16 that were found to be consistently over expressed in the Ts1Cje mice as compared to euploid mice, euploid genes from chromosomes other than 16 were also found to be differentially expressed at P0, P15 and P30. A first statistical analysis of the data using VARAN selected a total of 95 genes with ratios between Ts1Cje and euploid ranging from 0.1 to 0.8 and from 1.2 to 70.7. Five genes were from the triplicated segment of chromosome 16 and 90 were euploid genes. Table 3 displays the number of genes differentially expressed deduced from the 4 independent analysis at each time point (see Data analysis in Materials and Methods). Overlaps between the four analyses gave the final number of differentially expressed genes: 13, 57 and 25 at P0, P15 and P30 respectively (Table3). These genes are listed in Table 4. No differentially expressed genes were in common for all three developmental stages studied. However 3 genes were in common between P0 and P15 and three other genes between P15 and P30 (in bold in Table 4).

A second statistical analysis, ANOVA, was applied to the data. Table 5 gives the number of genes that are differentially expressed (over or under) and the test of the nullity of each parameter for several values of the risk of type I error (1%, 0.1% and 0.01%). For a 1% risk the number of genes differentially expressed is much higher between the stages of development (24.5% and 27.5% between P0 and P15 and between P15 and P30 respectively) than between the trisomic versus euploid state (5.1%, 4.1% and 3.1% at P0, P15 and P30, respectively). A complete list of genes is included in supplemental data table S4. All genes differentially expressed from VARAN were also detected by ANOVA and corresponded to the short list in Table 4, indicating that analysis with VARAN is more stringent than ANOVA. At P0, 86 and 154 genes had ratios above 1.5 and below 0.7 respectively. At P15, more genes had ratios below 0.7 than above 1.5 (154 and 86 respectively). At P30, 63 and 69 genes had ratios above 1.5 and below 0.7 respectively. Thirty one genes were in common between all three stages (Supplemental data Table S4). Fig.3 represents gene expression ratios between Ts1Cje and euploid mice with respect to the value of their statistic (Fisher) and shows that variations are more significant (higher Fisher) at P0 and P15 than at P30. Differentially expressed genes are either up- or down-regulated and nearly all triplicated genes belong to the up-regulated genes.

Classification of the differentially expressed genes from ANOVA according to gene ontology showed a significant enrichment only at P0 for GO categories GO:0007275 (p=0.00079) and GO:0030154 (p=0.00051), corresponding to genes involved in development and cell differentiation, respectively. Among the 419 differentially expressed genes at P0 (Table 5

Ts1Cje/euploid at P0 with α=1%) there were 297 genes with annotated ontology of which 6 and 17 were involved in cell differentiation and development respectively. Gene ontology is listed in a separate column in supplemental data table S4.

To confirm the reliability of microarray data, 7 differentially expressed genes from the short list of 95 genes selected by VARAN and ANOVA were analyzed by qPCR at P0, P15 or P30 (Table 6). From a total of 8 experiments conducted across three developmental stages, 6 variations were confirmed. Differential expression of *glutamine synthase* was found to be different when measured by qPCR as compared to microarray thus suggesting a lack of specificity of the microarray probe set.

Principal Component Analysis (PCA):

For gene profiling across all conditions, trisomic and euploid at P0, P15 and P30, we performed PCA. With the 8287 genes detected on the Affymetrix U74Av2 microarray the projection onto the subspace spanned by the two first principal components clearly grouped the animals according to their stage of development, thus demonstrating that the effect of development on gene expression is more important than the trisomic effect (Fig. 4A). PCA was then performed on subsets of genes of increasing size, the genes being ranked according to their discriminatory power for trisomy using Student statistic on data from either euploid or trisomic mice whatever their stage of development (see Materials and Methods). For the first 100 genes, PCA grouped the animals according to trisomy versus euploid state (Fig. 4D). However on larger subsets (3000 genes), animals were grouped according to their developmental stage (Fig. 4B). With 3000 genes, within each stage of development, Ts1Cje animals could be distinguished from the euploids. The use of 2000 genes resulted in an intermediate situation where the most likely separation was made according to the trisomic versus euploid state although one could also still get a separation according to the stage of development (Fig. 4C).

Comparison of microarray data from the literature:

Our data set was compared to those of two microarray studies published recently: that of Saran *et al.* (6), performed on adults (3-4 months) cerebellum from Ts65Dn mice, another mouse segmental trisomy 16 with a larger trisomic fragment (*App* to *Znf295*), and that of Amano *et al*.(7) performed on whole brains from Ts1Cje mice at P0. To compare all three studies, the data sets were represented the same way, namely the value of the statistic (t-test) with respect to the expression ratio of trisomic versus euploid (Fig. 5). For the study of Saran *et al.* (6) only the pools were analyzed. For our study, trisomic and euploid animals were

grouped together whatever their stage of development, and a t-test was applied. During postnatal development of the cerebellum in Ts1Cje mice (our study), there were genes differentially expressed (up and down), some from the triplicated region (7 over 29 detected on microarray) and some from the euploid region (44 over 8258) (Fig. 5A). In whole brain at P0 in Ts1Cje mice, most of the differentially expressed genes were up-regulated, with mainly genes from the triplicated region (25 over 45 and 76 over 10557 for the triplicated and euploid genes, respectively) (Fig. 5B). Finally in the cerebellum of adult Ts65Dn mice (3-4 months) there were genes up- and down-regulated, with a large proportion from the triplicated region (10 over 20 and 337 over 6581 for the triplicated and euploid genes, respectively) (Fig.5C). This comparative analysis revealed that the percentage of differentially expressed genes varies among studies, from 0.61% (our study when pooling the three time points) to 0.95% from Amano *et al.* (7), 5.35% in Saran *et al.* (6) and 5.1%, 4.1% and 3.1% (our study at P0, P15 and P30 respectively Table 5 and Fig.3) with a constant higher proportion of triplicated up-regulated genes as compared to euploid up-regulated genes.
Using the same statistical analysis of microarray data (VARAN), three genes/ESTs from the euploid genomic region were found to be differentially expressed in cerebellum of both Ts1Cje at P30 (our study) and Ts65Dn mice at 3-4 months (6): one repressed (MGI 96021 corresponding to hemoglobin β1 major chain) and two over expressed (Riken cDNA 1810007M14 and probe set Affymetrix 98525 corresponding to C21orf66 and to erythroid differentiation regulator 1 *Erdr1*, respectively).

Discussion

Gene-dosage effect of the triplicated genes from mouse chromosome 16 (MMU16) during postnatal development of the cerebellum in Ts1Cje mice:
Study of the cerebellar transcriptome during postnatal development in the Ts1Cje model of Down syndrome clearly shows an over expression of the three copy-genes, with mean ratios over postnatal development of 1.43 and 1.52 as determined by microarray and qPCR experiments, respectively. These results are in agreement with other studies published recently (6,7,18,19), thus demonstrating that there is a consistent modification in the number of transcripts throughout development resulting from a gene-dosage effect. From qPCR, not all genes had a 1.5 expression ratio between Ts1Cje and euploid, thus suggesting that there might be subtle compensatory mechanisms to the gene-dosage effect as already discussed previously (18,19). Gene-dosage effects on the genome and transcriptome has been described

in tumors (20,21), but there are genes for which the level of expression is not strictly dependant on the gene copy number, again suggesting the existence of compensatory mechanisms (22).

Development has a greater effect than trisomy on global gene expression:

Global gene expression profiling during postnatal development of the cerebellum enables mice to be classified according to their stage of development rather than to their trisomic versus eusomic status. This indicates that development has more impact on the expression profile than does gene-dosage (Fig. 4A). Furthermore, gene expression profiles of Ts1Cje cerebellum at P15 and P30 were not closer to the euploid animals at P0 and P15, respectively, thus indicating that there was no developmental delay in Ts1Cje mice (Fig 4A). Fig. 3 showed that there were more genes differentially expressed with significant statistical values at P0 and P15 than at P30. In addition, qPCR on triplicated genes indicated that there was a highly significant correlation ($p<0.01$) between the P15 and P30 time points but not between P0 and P15 or P30, suggesting that the developmental changes occurred between P0 and P15, and then stabilized.

Gene expression dysregulation of euploid genes:

Statistical analysis revealed a group of dysregulated genes with ratios ranging from 0.1 to 0.8 and 1.2 to 70.7 (short list in Table 4). The analysis was validated by qPCR on a group of 7 genes, the differential expression of 6 were confirmed. Comparative analysis of microarray data (our study on Ts1Cje and that of Saran *et al*. (6) on the cerebellum of adult Ts65Dn mice) revealed euploid genes that are severely differentially expressed (up or down) with large statistical values (Fig. 5A, 5C). In the transcriptome study on whole brain from Ts1Cje at P0, most differentially expressed genes were from the triplicated genomic segment, with ratios below 2 ((7) and Fig. 5B). Because total brain is a mixture of many different cell types, including cerebellar cells, cell specific regulation is diluted and below the level of detection. Reducing cellular complexity by the use of cerebellum increases the chance of seeing genes that are differentially expressed in a particular cell type. Using even smaller brain structures may reveal differentially expressed genes that are specific for one region of the brain.

The higher number of dysregulated genes found in our study (5.1%, 4.1% and 3.1% at P0, P15 and P30 respectively, Table 5) and in the study of Saran *et al.* (6) in adult Ts65Dn cerebellum (5.35%) as compared to 0.95% in the study of Amano *et al.* (7) on whole brain of Ts1Cje at P0 could relate to the complexity of the tissue used. In a similar way, pooling the

same tissue (cerebellum) at different time points (P0, P15 and P30) decreased the number of differentially expressed genes to 0.61% (Fig.5A). From VARAN analysis 2 euploid genes were selected as differentially expressed both from our study (Ts1Cje at P30) and from the data of Saran *et al.* (6) (Ts65Dn at 3-4 months). One, corresponding to hemoglobin β1 chain, was repressed, and the other, corresponding to erythroid differentiation regulator, was over expressed. Supplemental data table S4 shows that several hemoglobin α and β subunits were highly repressed at P30 and P15, thus suggesting a decrease in the amount of meninges that surround the cerebellum. Additional experiments done on animals bred on the same genetic background and at the same time points will be necessary to draw a final conclusion on particular genes.

Pathways modified during postnatal development of the cerebellum in Ts1Cje mice: cell differentiation and development:

We used gene ontology to categorize the differentially expressed genes from our microarray experiments and found that at P0 there was an enrichment of genes involved in cell differentiation and development. This suggests that cell differentiation and migration of cells might be more affected by trisomy than cell proliferation. At P0, of the 17 genes known to be involved in development, 6 are homeobox genes, all of which were repressed. In addition, at P15 and P30, respectively, 12 and 1 homeobox genes were found to be differentially expressed. Homeoproteins are involved in the early patterning of the nervous system and possibly at later stages of neuronal differentiation (23). Among them, *Homeo box A5* was found to be severely down-regulated (to 0.12) at P0 in our study as well as in two pools of cerebellum from adult Ts65Dn mice (0.47 and 0.54) (6). In the Amano *et al.* study (7) on whole brain of Ts1Cje mice, *Homeo box A5* was not detected. *Homeo box A5* is mostly expressed during the neonatal period, but there are studies relating to its expression in the adult, particularly in Purkinje cells, where it activates the transcription of Purkinje cell protein 2 (24).

At P15, *Dlx1* was found to be over expressed in Ts1Cje as compared to euploid mice (Table 6: 80 fold and 70.8 fold by microarray and qPCR, respectively). This very high ratio probably indicates that *Dlx1* is expressed only in trisomic cerebellum. *Dlx1* is not known to be involved in the postnatal development of the cerebellum. However, its role in the lineage of a particular type of GABAergic interneurons in human neocortex has been demonstrated recently (25). In addition, an increase of *Dlx1* and *Dlx2* negatively regulates *Notch* signalling to specify a later subset of neuronal progenitors and promote their terminal differentiation.

(26). *Dlx2* was also found to be increased at P15 (7 fold supplemental data table S4). It is therefore likely that increase of *Dlx1* and *Dlx2* may be associated with cell differentiation. We cannot exclude significant changes in the expression of transcription factors important for development of the cerebellum, such as *Math1, Mash1 and Shh,* that have been described in microarray studies (14), since they were not present on the Affymetrix U74Av2 microarray. More data on these particular genes will be useful for understanding cerebellum development, since *Math1* has recently been shown to be involved in the control of cerebellar granule cell differentiation by regulating multiple components of the *Notch* signalling pathway (27). Study of gene expression of *Notch* receptors and ligands and transcription factor targets will confirm if the *Notch* pathway is altered in Down syndrome.

Gene profiling from the study of Diaz *et al.* (14) on cerebellum mutants (*weaver* and *lurcher*) were compared to gene profiling in Ts1Cje mice using the same statistical analysis (ANOVA with the parameterisation described in Materials and Methods). In *weaver* mice granule cells are severely reduced in the internal layer of the adult cerebellum while in *lurcher* mice Purkinje cells are reduced (28,29). Comparison of Ts1Cje, *weaver* and *lurcher* revealed more similarity between Ts1cje and *weaver* mutants than between Ts1Cje and *lurcher* mutants (unpublished data). This is consistent with morphological data (13) suggesting that granule, rather than Purkinje cells, are altered in Ts1Cje mice.

The list of genes differentially expressed also includes transthyretin, a transporter of thyroid hormone and vitamin A, which has been shown to be a carrier of amyloid beta peptide in the cerebrospinal fluid. Transthyretin prevents formation of amyloid fibrils and Apo-E- induced accumulation of amyloid beta (30,31). In Ts1Cje mice, transthyretin was repressed on microarrays and by qPCR at P0 (Table 6). Repression of transthyretin, if present in Down syndrome, might contribute to the amyloid beta deposition process in brain in addition to APP over expression.

Another gene expression study in Down syndrome has shown that REST (neuron specific silencer factor) and its targets (SCG10, L1, synapsin) are down-regulated in neuronal precursor cells from Down syndrome fetal cortex (32). From our study, expression of SCG10, synapsin and L1 but not of REST was detected during postnatal development of the cerebellum. Only L1 was found to be differentially expressed, with a ratio of 0.52 between Ts1Cje and euploid at P0, although with low statistical value (Fisher=4.6 under the 1% threshold). Our data do not, therefore, provide evidence for or against the hypothesis of down regulation of REST pathway in neuronal precursor cells proposed by Bahn *et al.* (32).

In conclusion, in Ts1Cje mouse model of Down syndrome, the presence of three copies of approximately 85 genes having orthologues on human chromosome 21 induces a consistent change in expression of these triplicated genes throughout postnatal development. In addition to this general gene-dosage effect, where the expected 1.5 over expression ratio is largely but not totally respected, a number of euploid genes are differentially expressed in a time-dependant manner, with an over representation of transcripts involved in cell differentiation and development. Lastly, gene profiling reveals that development has more impact on the transcriptome than the trisomic/eusomic status and that there is no detectable delay in the postnatal development of the cerebellum of the segmental trisomy Ts1Cje mice.

Materials and methods:

Mice

Ts1Cje mice carry a segmental duplication of the MMU16 region from *Sod1* to *Znf295*. Ts1Cje mice were bred on a C57BL/6 background (11 backcrosses). For gene expression analysis, three pairs of euploid (C57BL/6) and trisomic Ts1Cje males sibs from two litters were used for each developmental stage P0, P15 and P30.

Genotyping

Because the trisomic segment of MMU16 has a truncated *Sod1* allele with the neomycin resistance cassette, the mice were first typed on genomic DNA from the tail by PCR using *Neo* primers (10).

In addition, after individual RNA extraction (see below), all animals were tested for the presence of both *Sod1* wild type and truncated alleles by PCR on cDNAs using *Sod1* primers: up-CAATGTGACTGCTGGAAAGG and low-ATCCCAATCACTCCACAGGC. For each mouse, 500ng of total RNA from cerebellum were reverse-transcribed overnight at 37°C in the presence of 200 units of Superscript II (InVitrogen), 1x first-strand buffer, 100µM DTT, 5µM random hexamers oligonucleotides (Pasteur Institute) and 500µM dNTPs. PCRs were performed using 2.5 units of HotstartTaq® DNA polymerase (Qiagen) with 1x PCR buffer containing 1.5mM $MgCl_2$, 0.3µM of each primer and 200µM dNTPs for 40 cycles of 94°C for 30s, 58°C for 30s, 72°C for 40s. Because one of the P30 euploid mice showed a pattern with an unexpected number of bands, we decided not to use it, and the euploid P30 pool was therefore composed of only 2 animals instead of 3.

RNA extraction and microarray hybridizations

Total RNA was extracted from frozen individual cerebellum and treated with DNAse using RNeasy Midi kit (Qiagen). The quality of each RNA sample was then checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA).

For the 3 postnatal stages, euploid and trisomic samples were prepared by pooling an equal amount of each individual RNA. 20µg of total RNA from each pool were converted to cDNA and then to biotinylated cRNA and were hybridized to Affymetrix Murine Genome U74A version 2 microarrays (Affymetrix, Inc., Santa Clara, CA, USA) on the Curie Institute Affymetrix microarray platform (Paris, France) according to Affymetrix procedures. Each pool was hybridized in duplicate on independent U74Av2 microarrays.

Data analysis: filtering, normalization, VARAN and ANOVA, ontology:

After hybridization, signal intensities were calculated using the Affymetrix GeneChip® software MAS 5.0. The software generates a "detection p-value" and a "detection call" to decide whether or not a gene is expressed, and then attributes to each gene a status: present, absent or marginal. For further analysis, we used only the 8287 genes that were defined as "Present" or "Marginal" in Ts1Cje or euploid mice in at least one time point. Genes called "Absent" in all conditions were not used for further analysis.

We have deposited all the raw data (Accession number GSE1611) in the NCBI public database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

Filtered data (8287 genes) were submitted to VARAN (http://www.bionet.espci.fr,) for normalization and differential expression analysis (16). The microarray measurements were grouped into 6 pairs of replicates: euploids at P0, P15, and P30, and Ts1Cje at P0, P15, and P30. The expression levels were corrected in order to constrain the mean ratio of each pairs to be equal to one using a Lowess fit on the M-A plot of the log-expression levels (16). In the numerical analysis, the logarithm of the expression levels was used. For each experiment replicates on euploid mice (i.e. at P0, P15 and P30), a reference M-A plot was established. Assuming that the variance of the gene expression level was a function of their mean, and that the expression levels were Gaussian, the 1% threshold values for the difference as a function of the mean were estimated.

For each stage, the M-A plots of the 4 possible combinations of euploid and Ts1Cje mice were established thus providing 4 analysis at each time point, and the genes whose difference of expression between euploid and trisomic exceeded the 1% threshold values were selected.

For the ANOVA, we applied a regression model for gene expression levels. Table 1 below indicates the parameters $\{\alpha_i\}$ that model the effects of development in euploid mice, and the $\{\beta_i\}$ that model the effects of trisomy at each development stage. The number n of parameters equals 6, and the number N of microarray measurements equals 12.

	P0	P15	P30
Euploid	μ	$\mu + \alpha_2$	$\mu + \alpha_3$
Ts1Cje	$\mu + \beta_1$	$\mu + \alpha_2 + \beta_2$	$\mu + \alpha_3 + \beta_3$

Table 1 Parameters of gene expression levels.

Assuming a Gaussian distribution of gene expression levels with common variance s^2, the sum of squared residuals of the model were χ^2 distributed: $SSR / \sigma^2 \rightarrow \chi^2(N - n)$. If trisomy

had no effect at a given stage we assumed the nullity of the corresponding parameter (null hypothesis H_0). If H_0 is true, $(SSR_0 - SSR)(N-n)/SSR \rightarrow Fisher(1,N-n)$, where SSR_0 corresponded to the sum of squared residuals of the associated submodel. Hence the test: reject H_0 if the Fisher statistic $(SSR_0 - SSR)(N-n)/SSR > f_\alpha(1,N-n)$, where α denoted the chosen significance level, and $f_\alpha(1, N-n)$ the corresponding quantile of the Fisher distribution.
Gene ontology: The hypergeometric distribution was used to calculate the probability of observing individual GO terms as enriched in sets of differentially expressed genes relative to the reference set of expressed genes on the Affymetrix U74Av2 microarray.

Principal Component Analysis (PCA):

PCA was first performed on the 8287 expressed genes and then on subsets of genes of increasing size, the genes being sorted by their discriminatory power for trisomy. The discriminatory power of gene i was measured by the absolute value of its Student statistic t^i:

$$t^i = \frac{\overline{x^i_{Ts}} - \overline{x^i_{Eu}}}{s^i \sqrt{\frac{1}{N_{Ts}} + \frac{1}{N_{Eu}}}}$$

where $N_{TS} = N_{Eu} = 6$ was the number of measurements on trisomic and euploid mice respectively whatever their stage of development, $\overline{x^i_{Ts}}$ and $\overline{x^i_{Eu}}$ the mean expression of gene i for the trisomic and euploid mice respectively, and s^i the intraclass variance estimate.

Real-Time Quantitative PCR (qPCR)

250ng and 6µg of total RNA from each euploid and trisomic pool were individually converted into cDNAs overnight at 37°C in the presence of 200 units of Superscript II (InVitrogen), 1x First-strand buffer, 100µM DTT, 5µM random hexamers oligonucleotides (Pasteur Institute) and 500µM dNTPs.
The cDNAs were then diluted 1:5 and 1:14 for the 250ng and 6µg RNA samples, respectively, for qPCR according to the following protocols:qPCR on the 78 MMU16 genes and 18 control genes mapping to chromosomes other than 16 were performed as Taqman assays in an ABI 7900 Sequence Detection System (Applied Biosystems). Assay sequences are available in Lyle et *al*. (18). Each expression value corresponds to the mean of 5 replicates.
Quantitative PCR on the 7 genes outside MMU16 that were found to be differentially expressed by microarray analysis (*ALDR, Astrotactin, Dlx1, Glutamine synthase, Homeo box*

A5, transthyretin, Uncx4.1) were performed in a Lightcycler system (Roche Molecular Biochemicals), in the presence of 0.5µM of each specific primer (designed by Oligo4 software) and 1x Quantitect™ SYBR® Green PCR master mix (Qiagen) containing 2.5mM $MgCl_2$, HotstartTaq® Polymerase, dNTP mix and the fluorescent dye SYBR Green I. Each reaction was performed in triplicate. The list of primers is given in Supplemental data table S5.

For each sample tested, 3 control genes, *GAPD, tubulin and Ppox* that showed no difference between Ts1Cje and euploids were used to normalize raw data, and the mean relative ratios Ts/Eu were calculated according to geNorm (33) as described in Lyle *et al.* (18).

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Figure legends:

Fig. 1: Distribution of gene expression ratios between Ts1Cje and euploid mice in cerebellum at postnatal day 0 (P0), 15 (P15) and 30 (P30) using mouse genome U74Av2 Affymetrix microarray data. Genes are subdivided as euploid genes from or outside mouse chromosome 16 (MMU16) and trisomic genes from MMU16.

Fig. 2: Differences in the expression of 78 genes from mouse chromosome 16 (MMU16) and 18 control genes outside MMU16 between Ts1Cje and euploid mice in cerebellum at P0, P15 and P30 by real time qPCR. Genes are ordered according to their chromosomal location (MMU16 or non MMU16, for the MMU16 genes the order along the chromosome is respected) and their copy number (euploid or trisomic). Mean expression ratios (n=5) are indicated at the bottom.

Fig. 3: Statistical analysis of genes from microarray data differentially expressed between Ts1Cje mice and euploid mice at P0 (A), P15 (B) and P30 (C). Asterisks, crosses and open circles correspond to MMU16 trisomic genes, MMU16 euploid genes and non MMU16 euploid genes respectively. ANOVA (α=1%, n=6, N=12, see Materials and Methods).

Fig. 4: PCA on 8287 expressed genes from mouse genome U74Av2 Affymetrix microarrays at P0, P15 and P30 (A) and on subsets of genes ranked according to trisomic versus euploid state using a t-test: the first 3000 genes (B), 2000 genes (C) and 100 genes (D), Open and filled symbols correspond to euploid and trisomic mice respectively. Circles, triangles and lozenges correspond to P0, P15 and P30 respectively.

Fig. 5: Comparison of differentially expressed genes in three studies: in Ts1Cje cerebellum at P0, P15 and P30 (our study, A), in whole brain Ts1Cje at P0 (Amano *et al.* (7), B), in Ts65Dn cerebellum at 3-4 months (Saran *et al.* (6), C). For comparing the three studies a t-test was applied to all three sets of data.

Table 1: Parameters of gene expression levels for the ANOVA.

Table 2: List of triplicated genes found to be differentially expressed by ANOVA of microarray data. Comparison with VARAN (yes indicates that the gene was selected with VARAN), (16) and qPCR (mean values of ratios are indicated). Ts: trisomic; Eu: euploid.

Table 3: Number of differentially expressed genes among the 8287 genes detected on U774Av2 Affymetrix microarray according to VARAN (1% threshold). The short list (95 genes) is given in Table 4. [1] and [2] indicate replicate experiments on pools of cerebellum.

Table 4: List of genes whose expression is changed in Ts1Cje mice as compared as to euploids according to both VARAN and ANOVA. In grey are genes from the triplicated genomic segment from mouse chromosome 16. In bold are genes that were shown to be dysregulated at 2 time points.

Table 5: Number of differentially expressed genes among the 8287 at P0, P15 and P30 according to ANOVA (rejection rate of H_0 see Materials and Methods).

Table 6: Comparison between Ts1Cje/euploid expression ratios of genes selected by VARAN (16) obtained either by qPCR or from the Affymetrix U74Av2 microarray data.

Supplemental data table S1: List of the 8282 genes found to be expressed at either P0, P15 or P30 in the cerebellum of Ts1Cje or euploid mice from the Affymetrix U74Av2 data. ANOVA analysis.

Supplemental data table S2: List of genes differentially expressed between P0 and P15 (alpha2) and between P15 and P30 (alpha3) in either euploid or Ts1Cje mice. Gene classification according to Diaz *et al.* (14) is given in the table.

Supplemental data table S3: Data from qPCR on 78 genes from MMU16 (15 euploid and 63 trisomic) and 18 control genes mapping to chromosomes others than MMU16.

Supplemental data table S4: Genes differentially expressed between Ts1Cje and euploid mice at P0, P15 and P30 selected by ANOVA and VARAN. Overlaps between 2 or 3 time points.

Supplemental data table S5: List of PCR primers used in qPCR in Table 6: *ALDR, Astrotactin, Dlx1, Glutamine synthase, Homeo box A5, transthyretin, Uncx4.1*

Abbreviations

QPCR quantitative polymerase chain reaction; SAGE serial analysis of gene expression; *Sod1* superoxyde dismutase 1; *Znf295* zinc finger protein 295; ANOVA analysis of varaince; EST expressed sequence tag; VARAN variability analysis of microarray data; *Dscr* down syndrome critical region; *HMGP* high mobolity group nucleosomal binding domain protein; *Donson* downstream neighbor of SON; *C21 orf* chromosome 21 open reading frame; PCA principal component analysis; *Erdr1* erythroid differentiation regulator;

Gene (probe set)	Stage	Ts/Eu ratio U74Av2	Fisher	VARAN selected	Ts/Eu ratio qPCR
Atp5o (99128 at)	P15	1.70	29.4	yes	1.42
Atp5o (99128 at)	P30	1.45	15.1		1.39
C21orf4 (96340 at)	P0	1.48	69.5		1.88
C21orf5 (96340 at)	P15	1.62	110.8		1.45
C21orf6 (96340 at)	P30	1.41	57.6		1.52
Donson (94386 at)	P0	1.91	97.1		1.66
Donson (94386 at)	P15	1.45	30.5		1.38
Donson (94386 at)	P30	1.78	76.1	yes	1.38
Dscr1 (100555 at)	P0	1.48	29.4		1.62
Dscr1 (100555 at)	P15	1.74	63.3		1.49
Dscr3 (101104 at)	P0	1.32	14.3		1.39
Dscr3 (101104 at)	P15	1.58	40.6		1.45
Dscr3 (101104 at)	P30	1.48	29.5		1.39
Dscr3 (162486 f at)	P0	1.70	18.4		1.39
Ets2 (94246 at)	P0	1.23	14.5		1.45
Ets2 (94246 at)	P30	1.29	21.4		1.78
Gart (100066 at)	P0	1.45	34.9		1.43
Gart (100066 at)	P30	1.55	51.7		1.53
GCR-DNABF[a] (92233 at)	P0	1.55	38.6		
GCR-DNABF[a] (92233 at)	P30	1.70	58.3	yes	
Hmgp14 (96699 at)	P0	1.58	87.9	yes	1.84
Hmgp14 (96699 at)	P15	1.35	35.4		1.19
Hmgp14 (96699 at)	P30	1.29	28		1.33
Ifnar1 (100483 at)	P30	2.04	28	yes	1.64
Il10rb (99491 at)	P0	1.32	15.2		1.42
Myo5a (160899 at)	P0	1.51	17.7		1.84

Table 2

	P0	P15	P30
Euploid[1] /Ts1Cje[1]	100	166	203
Euploid[1]/ Ts1Cje[2]	179	377	283
Euploid[2]/ Ts1Cje[1]	330	225	261
Euploid[2]/ Ts1Cje[2]	259	248	233
Differentially expressed genes	13 (0.16%)	57 (0.69%)	25 (0.30%)

Table 3

Table 4

Gene	Probe set	Stage	Ts/Eu ratio U74Av2	Fisher
adenosine deaminase	98632_at	P15	3.31	1.9
adenylate cyclase 6	102321_at	P15	1.95	69.4
aminolevulinic acid synthase 2, erythroid	92768_s_at	P15	0.30	50.7
astrotactin 1	92702_at	P15	0.23	14.9
ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	99128_at	P15	1.70	29.4
ATPase, Na+/K+ transporting, alpha 1 polypeptide	93797_g_at	P0	0.44	63.4
carbonic anhydrase 2	92642_at	P30	0.63	42.5
cathepsin C	101019_at	P15	4.47	75.3
CD151 antigen	97932_f_at	P15	2.00	28.9
chromogranin B	92841_f_at	P15	0.71	28.8
copine 6	93151_at	P15	6.61	51.3
cytoplasmic polyadenylation element binding protein	160680_at	P15	1.55	0.6
distal-less homeobox 1	98394_at	P15	70.79	181.8
DNA segment, Chr 6, ERATO Doi 109, expressed	96754_s_at	P15	1.86	62.8
ELKL motif kinase	99458_i_at	P30	1.70	64.1
eomesodermin homolog (Xenopus laevis)	93880_at	P30	2.45	10.5
EST	99849_at	P0	1.58	33.9
EST	96731_at	P15	2.09	111.2
EST	94106_at	P15	1.91	311.3
EST	96755_at	P15	1.91	9
EST	161050_at	P15	1.58	29.5
EST	98594_at	P15	0.59	12.4
EST	96215_f_at	P15	0.49	29.1
EST	104477_at	P15	0.21	79
EST	103432_at	P15	0.11	19.8
EST	98525_f_at	P30	1.78	95
EST	103211_at	P30	0.48	5.2
expressed sequence AA408420	96541_at	P15	1.86	70.2
expressed sequence AI467657	92202_g_at	P30	5.13	157.8
expressed sequence AI551766	104235_at	P15	2.19	43.4
expressed sequence AI852661	95397_at	P15	1.86	20.1
expressed sequence C80126	103295_at	P30	0.54	47.7
expressed sequence C87222	101929_at	P15	1.41	100.4
fatty acid binding protein 7, brain	98967_at	P30	0.46	52.8
FK506 binding protein 12-rapamycin associated protein 1	104708_at	P30	2.14	20.6
FK506 binding protein 5 (51 kDa)	94297_at	P30	2.69	58.7
glutamate receptor, ionotropic, NMDA2C (epsilon 3)	97726_at	P15	0.56	1.9
glutamine synthetase	94852_at	P0	0.45	365.3
glutamine synthetase	94852_at	P15	2.69	552.4
hemoglobin alpha, adult chain 1	94781_at	P15	0.39	217.2
hemoglobin alpha, adult chain 1	94781_at	P30	0.63	50.7
hemoglobin, beta adult major chain	101869_s_at	P15	0.49	686.9
hemoglobin, beta adult major chain	162457_f_at	P15	0.32	65.9
hemoglobin, beta adult minor chain	103534_at	P15	0.38	93
hemoglobin, beta adult minor chain	103534_at	P30	0.38	90.5
high mobility group nucleosomal binding domain 1	96699_at	P0	1.58	87.9
homeo box A5	103086_at	P0	0.13	396.2
homeo box B5	103666_at	P0	0.17	45
homeo box D4	102380_s_at	P0	0.18	21.6

hydroxysteroid 17-beta dehydrogenase 11	102370_at	P15	1.78	81
immunoglobulin superfamily, member 4	93604_f_at	P30	1.66	119.2
inactive X specific transcripts	99126_at	P15	38.90	57.4
interferon (alpha and beta) receptor	100483_at	P30	2.04	28
LPS-responsive beige-like anchor	104264_at	P15	1.86	95.3
Max dimerization protein 4	99024_at	P15	1.95	194.4
Max dimerization protein 4	99024_at	P30	1.70	117.1
metallothionein 1	93573_at	P30	1.78	35.6
metallothionein 2	101561_at	P30	1.95	64.4
midnolin	104410_at	P15	1.95	152.9
mitogen activated protein kinase 8	104047_at	P0	0.56	184.2
mitogen activated protein kinase 8	104047_at	P15	2.00	250.6
myelin and lymphocyte protein; T-cell differentiation protein	99089_at	P15	1.86	35.1
myeloid ecotropic viral integration site-related gene 1	97988_at	P15	9.77	32
neurofilament, medium polypeptide	92346_at	P15	2.19	29.1
nucleolar and coiled-body phosphoprotein 1	95735_at	P0	0.23	22.6
open reading frame 60	94386_at	P30	1.78	76.1
phosphatase and tensin homolog	160614_at	P30	0.59	13.4
prosaposin	97560_at	P30	0.55	49.2
protease, serine, 18	92353_at	P30	0.32	4.1
protein tyrosine phosphatase, receptor type, O	100427_at	P15	2.19	77.9
regulated endocrine-specific protein 18	99442_at	P15	2.45	27.7
ribosomal protein L29	94240_i_at	P0	0.06	15
ribosomal protein, large, P1	161480_i_at	P30	1.62	1.9
RIKEN cDNA 1500036F01 gene	160264_s_at	P15	2.34	52
RIKEN cDNA 1810007M14 gene	92233_at	P30	1.70	58.3
RIKEN cDNA 2610007K22 gene	101962_at	P15	1.41	147.7
RIKEN cDNA 2610528C18 gene	160868_at	P15	5.01	79
RIKEN cDNA 2810407C02 gene	97292_at	P15	1.78	8.4
RIKEN cDNA 2900073H19 gene	103624_at	P0	0.22	10.4
RIKEN cDNA 4933406L05 gene	161011_at	P15	0.48	8.1
RIKEN cDNA 4933407C03 gene	103748_at	P30	0.26	45.7
RIKEN cDNA 5930418K15 gene	102870_at	P0	1.95	361.4
RIKEN cDNA 5930418K15 gene	102870_at	P15	0.60	217.5
RIKEN cDNA 6330403K07 gene	95559_at	P15	2.00	130.5
RIKEN cDNA 9430059D04 gene	99591_i_at	P15	0.50	23.7
short stature homeobox 2	99042_s_at	P15	5.50	36.5
small inducible cytokine subfamily D, 1	98008_at	P15	2.29	98.4
somatostatin	95436_at	P15	3.09	21.2
thymus cell antigen 1, theta	99057_at	P15	1.51	47.2
transthyretin	95350_at	P0	0.39	304.2
tyrosine hydroxylase	100690_at	P15	2.75	36.1
Unc4.1 homeobox (C. elegans)	92499_at	P15	0.51	52.6
X-linked lymphocyte-regulated 3b	101883_s_at	P30	3.98	116.8
zeta-chain (TCR) associated protein kinase (70kD)	93662_s_at	P15	4.68	5.7
zinc finger protein 179	100383_at	P15	0.52	23

Table 5

			P0/P15	P15/P30	Ts/Eu at P0	Ts/Eu at P15	Ts/Eu at P30
α	$f_\alpha(1,6)$	m = 0	$\alpha_2 = 0$	$\alpha_3 = 0$	$\beta_1 = 0$	$\beta_2 = 0$	$\beta_3 = 0$
1%	13.745	7959 (96.0%)	2038 (24.6%)	2282 (27.5%)	419 (5.1%)	339 (4.1%)	257 (3.1%)
0.1%	35.51	7704 (92.96%)	1136 (12.50%)	1195 (14.42%)	105 (1.27%)	94 (1.13%)	61 (0.74%)
0.01%	82.489	7377 (89.0%)	506 (6.1%)	610 (7.4%)	26 (0.3%)	25 (0.3%)	14 (0.2%)

Table 6

	Ts/Eu at P0		Ts/Eu at P15	
GENE	**U74Av2**	**qPCR**	**U74Av2**	**qPCR**
ALDR	0.46	0.44		
Astrotactin			0.79	0.23
Dlx1			80	70.8
Glutamine synthase	1.09	0.45	1.1	2.69
Homeo box A5	0.12	0.13		
Transthyretin	0.12	0.39		
Uncx4.1			0.80	0.51

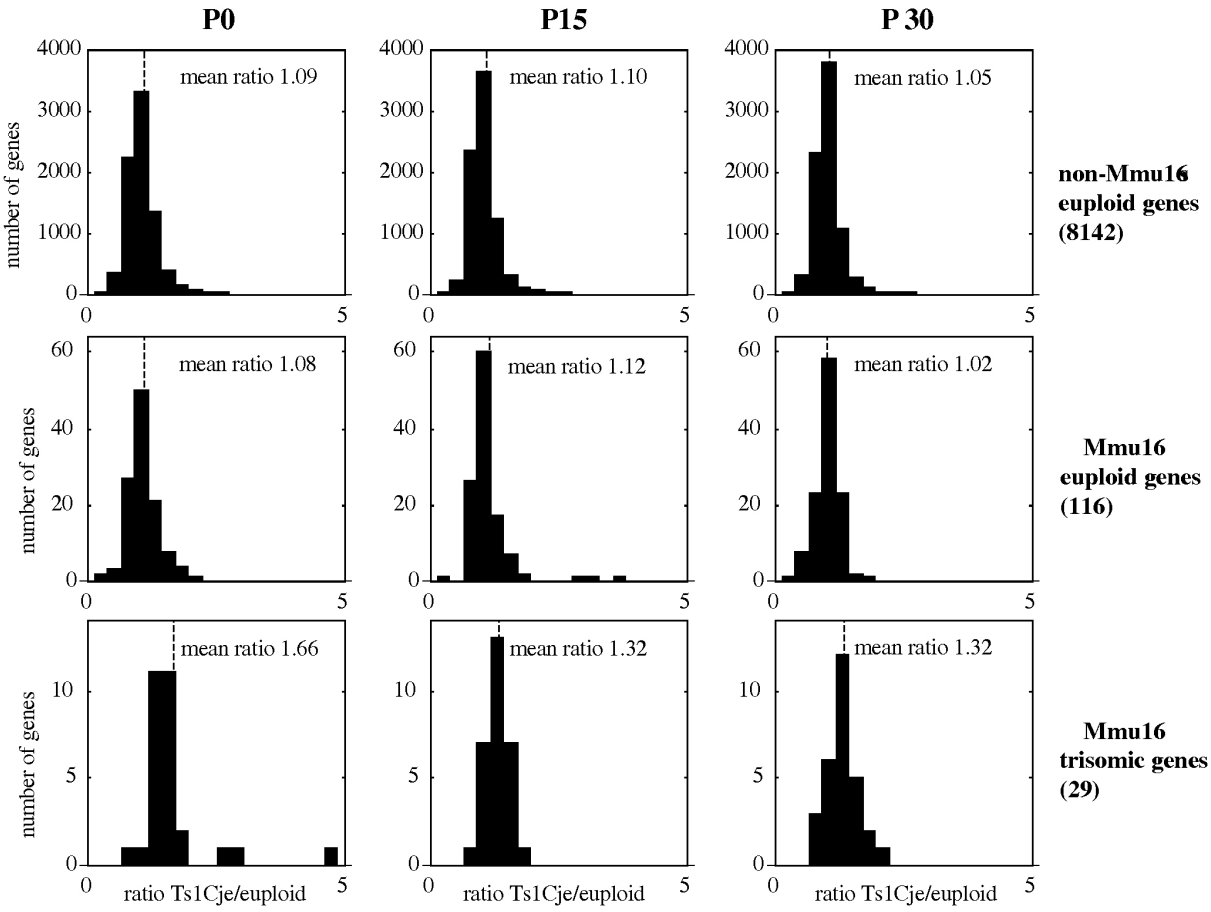
P0
P15
P 30
mean ratio 1.09
mean ratio 1.10
mean ratio 1.05
mean ratio 1.08
mean ratio 1.12
mean ratio 1.02
mean ratio 1.66
mean ratio 1.32
mean ratio 1.32
number of genes
ratio Ts1Cje/euploid
non-Mmu16 euploid genes (8142)
Mmu16 euploid genes (116)
Mmu16 trisomic genes (29)

Fig. 1

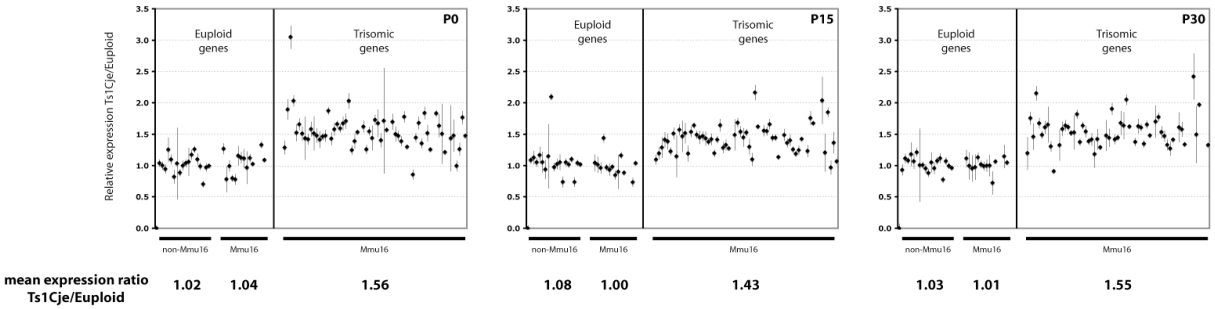
Relative expression Ts1Cje/Euploid
Euploid genes
Trisomic genes
P0
P15
P30
non-Mmu16
Mmu16
mean expression ratio Ts1Cje/Euploid
1.02
1.04
1.56
1.08
1.00
1.43
1.03
1.01
1.55

Fig. 2

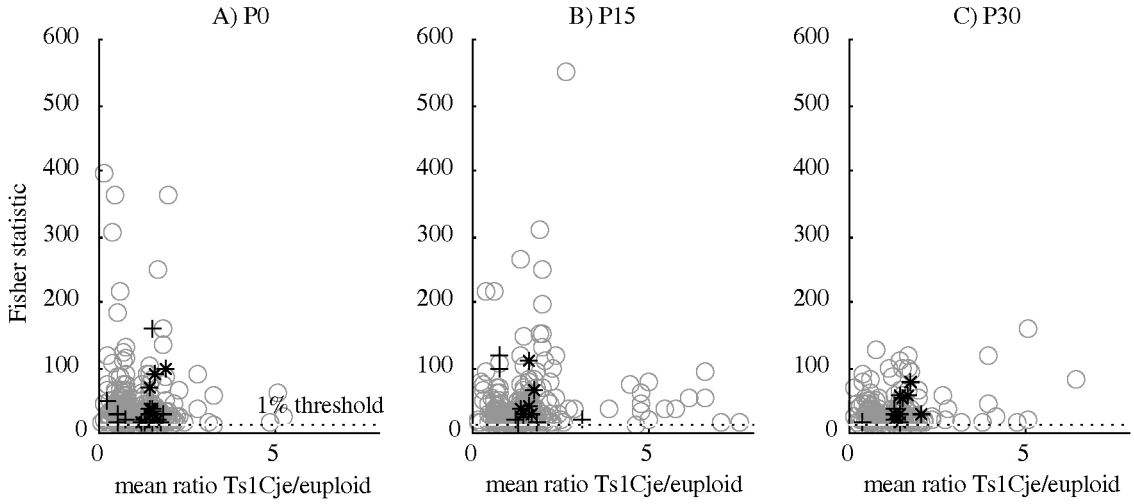
A) P0
B) P15
C) P30
Fisher statistic
1% threshold
mean ratio Ts1Cje/euploid

Fig. 3

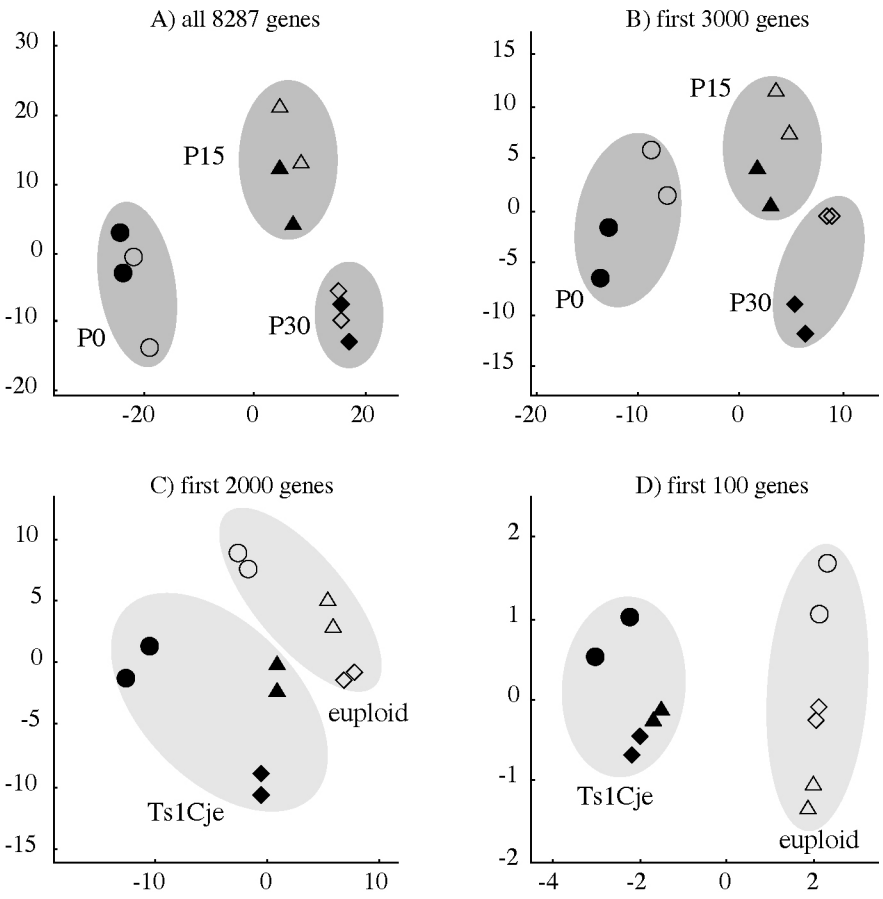
A) all 8287 genes
P15
P0
P30
B) first 3000 genes
P15
P0
P30
C) first 2000 genes
euploid
Ts1Cje
D) first 100 genes
Ts1Cje
euploid

Fig. 4

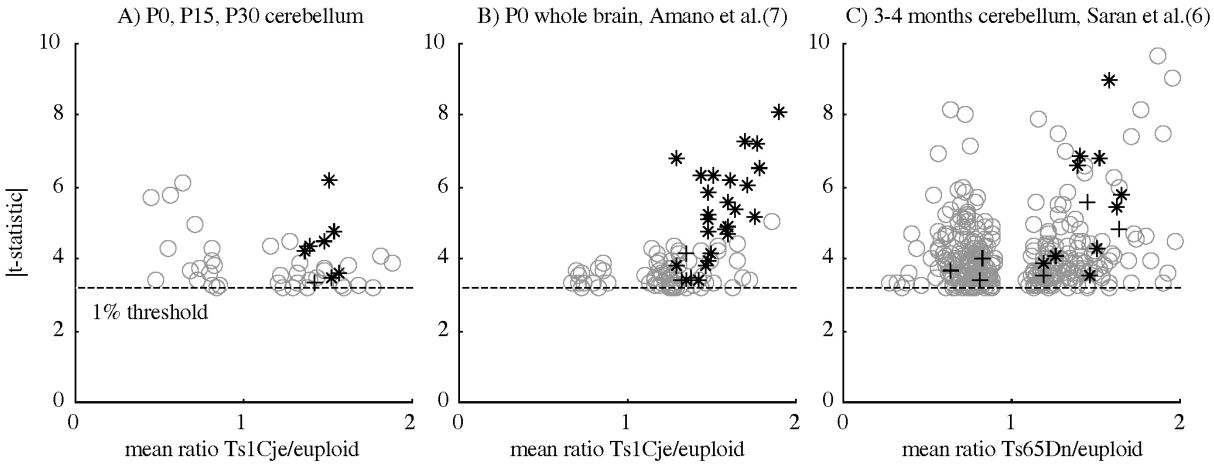
A) P0, P15, P30 cerebellum
B) P0 whole brain, Amano et al.(7)
C) 3-4 months cerebellum, Saran et al.(6)
|t-statistic|
1% threshold
mean ratio Ts1Cje/euploid
mean ratio Ts1Cje/euploid
mean ratio Ts65Dn/euploid

Fig. 5